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Novel Hepatitis E Virus (HEV) Isolates From Europe: Evidence for Additional Genotypes of HEV

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Hepatitis E infection is associated with areas in which hepatitis E virus (HEV) infection is endemic. Acute infections in industrialized nations are usually linked to travel to endemic areas. Recently, an acute hepatitis infection in a patient from the United States (US), with no recent foreign travel history, was linked to a novel strain of HEV. Although a few additional cases have been reported from patients who have not traveled to endemic areas, the source of these infections has not been determined. The objective of this study was to identify additional HEV isolates from patients with acute infection who had no recent history of travel to areas where HEV is considered endemic, and to determine the genetic relationship between these and other HEV isolates. Viral RNA was isolated from serum and polymerase chain reaction (PCR) was performed using consensus primers based on a number of HEV isolates. HEV sequence in open reading frame (ORF) 1 and ORF2 was identified in three patients from nonendemic areas, one from Italy and two from Greece. Comparative and phylogenetic analyses were performed. The Greek and Italian isolates were significantly divergent from two isolates from the US and isolates identified previously from HEV-endemic regions. The Italian isolate was distinct from the two Greek isolates. In addition, the two Greek isolates were significantly divergent from each other. Phylogenetic analysis indicated that the Italian and two Greek isolates represent three new genotypes of HEV, distinct from the Burmese, Mexican, and US genotypes. ***J. Med. Virol. 57:243–251, 1999.***

KEY WORDS: European strains; Greek isolate; Italian isolate; nonendemic isolates; non-A-C hepatitis; phylogenetic analysis

INTRODUCTION

Hepatitis E virus (HEV) has historically been referred to as enterically transmitted non-A, non-B hepatitis or "waterborne hepatitis." HEV is a major cause of epidemic hepatitis and acute, sporadic hepatitis in developing nations [Khuroo, 1980; Bradley 1990; Krawczynski, 1993; Mushahwar and Dawson, 1997]. HEV is a nonenveloped virus with a positive-sense, single-stranded RNA genome of approximately 7.2 kb. The virus is approximately 27–34 nm in diameter and has been classified tentatively as a member of the *Caliciviridae.* The viral genome consists of three discontinuous, partially overlapping open reading frames (ORFs). ORF1 encodes nonstructural gene products such as the helicase, protease, and RNA-dependent RNA polymerase proteins, ORF2 encodes the capsid protein, and ORF3 has been reported to encode a small phosphoprotein that associates with the cytoskeleton [Tam et al., 1991; Huang et al, 1992; Zafrullah et al., 1997].

The genomes of the three prototype strains of HEV from Burma, Mexico, and the United States (US) have been sequenced [Tam et al., 1991; Huang et al, 1992; Schlauder et al., 1998]. The overall nucleic acid sequence identity is 74–76% and the deduced amino acid identities are 82–84%, 90–93%, and 79–87%, for ORF1, ORF2, and ORF3, respectively [Erker et al., 1999]. Earlier studies indicated that HEV isolates identified from a number of outbreaks and sporadic cases appear to be closely related to the Burmese strain, with greater than 92% nucleotide identity across the genomes. However, analyses of HEV sequences reported recently in several sporadic cases from China [Huang et al., 1995; Hsieh et al., 1998; Wang et al., 1999; Wu et al., 1998] suggest that variants from endemic regions that are

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significantly divergent from the Mexican and Burmese strains do exist.

Whereas most hepatitis E infections have been linked to epidemics or to cases of acute, sporadic hepatitis from endemic areas, there have been occasional reports of HEV-associated hepatitis in areas where HEV is rare, such as the US and Western Europe. In most cases, these infections have been shown to be associated with travel to areas where HEV is endemic [Bader et al., 1991; Dawson et al., 1992a; Smalley et al., 1996]. Thus, clinical cases of HEV-associated hepatitis and HEV infections in general appear to be rare in industrialized countries. However, seroepidemiologic studies indicate that IgG-class antibodies to HEV proteins are detected in 1–2% of blood donors in the US and Western Europe [Dawson et al., 1992b; Paul et al., 1994]. Similar results have been reported recently by other investigators [Quiroga et al., 1996; Mast et al., 1997], suggesting that HEV infections are more widely distributed than originally believed and that additional strains of HEV may be circulating in industrialized nations. Several isolated reports of HEV-associated hepatitis in Europe and the US among individuals with no history of travel to regions traditionally considered endemic for HEV support this conclusion [Zaijer et al., 1993; Zanetti et al; 1994; Psichogiou et al., 1995; Kwo et al., 1997; Thomas et al., 1997].

The existence of novel strains of HEV from nonendemic regions was recently established with the identification of a native strain of HEV in the US [Kwo et al., 1997; Schlauder et al., 1998]. The close homology between an HEV-like sequence from swine herds in the US [Meng et al., 1997] and the novel HEV isolate from the US patient suggests a possibility of zoonosis [Schlauder et al., 1998]. Preliminary data have indicated that a novel isolate may also be native to Europe [Zanetti et al., 1999]. In this report, we provide evidence for the presence of additional novel strains of HEV isolated from patients in Europe who reported no travel to areas outside their respective countries.

MATERIALS AND METHODS

Source Material

Acute phase sera were obtained from three patients described previously in studies on the prevalence of HEV in Greece and Italy [Tassopoulos et al., 1994; Zanetti et al., 1999]. The patients had reported neither a history of travel to areas endemic for HEV, nor any contact with individuals who lived in endemic areas. All three patients had exhibited an acute phase HEV IgM response lasting from 4 to 6 weeks, as well as an HEV IgG response lasting as long as 3.5 years in one patient. Serum from the Italian patient was polymerase chain reaction (PCR) positive for HEV 7 days postpresentation (dpp) using primers based on the US isolate of HEV [Schlauder et al., 1998]. Initial sequence analysis has indicated that this isolate is probably distinct from other isolates of HEV [Zanetti et al., 1999]. One of the two patients from the study in Greece had also been found to be PCR positive [Psichogiou et al., 1995] using primers based on the Burmese isolate of HEV [Schlauder et al., 1993]; however, the nucleotide sequence of the product was not determined.

Reverse Transcriptase-PCR (RT-PCR)

Sequences were identified using RNA extracted from 25 to 50 μl of serum using the Ultraspec RNA Isolation System (Biotecx, Houston, TX) as described by the manufacturer. RT-PCR was performed using the GeneAmp RNA PCR Kit essentially according to the manufacturer's instructions (Perkin-Elmer, Norwalk, CT). RNA was reverse transcribed in the presence of random hexamers. PCR was performed with the cDNA encompassing one-fifth of the total reaction volume (10–25 μl) in the presence of 2 mM $MgCl_2$ and 0.5 μM of each primer. The degenerate PCR primers were designed within regions of identity between Burmese, Mexican, and US isolates [Erker et al., 1999; Wang et al., 1999]. The degenerate ORF1 primers, HEVConsORF1-s1, CTGGCATYACTACTGCYATTGAGC, positioned at nucleotides 56–79 (Burmese isolate numbering, Huang et al., 1992) and HEVConsORF1-a1, CCATCRARRCAGTAAGTGCGGTC, positioned at nucleotides 473–451, amplify a product of 418 base pairs. The degenerate primers from ORF2, HEVORF2con-s1, GACAGAATTRATTTCGTCGGCTGG, positioned at nucleotides 6,298–6,321, and HEVORF2con-a1, CTTGTTCRTGYTGGTTRTCATAATC, positioned at nucleotides 6,494–6,470, produce a product of 197 base pairs. Amplification involved 43 cycles of 94°C for 30 seconds, 55°C for 30 seconds (–0.3°C/cycle), and 72°C for 1 minute. This step was followed by 10 cycles of 94°C for 30 seconds, 40°C for 30 seconds, and 72°C for 1 minute. Amplified products were separated on a 1.5% agarose gel and examined for the presence of PCR products of the appropriate size.

Sequence Analysis

PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned with the Perfectly Blunt Cloning Kit into pT7-Blue T-Vector (Novagen, Madison, WI) according to the manufacturers' instructions. For each product, at least two clones were sequenced using a 373 DNA Sequencer (Applied Biosystems, Foster City, CA) with the Sequencing Ready Reaction Kit as specified by the manufacturer.

Nucleotide sequences were compiled using the program Sequencher, version 3.0 (GeneCodes, Ann Arbor, MI). Nucleotide and amino acid comparisons and alignments were performed with the GAP and PILEUP programs of the Wisconsin Sequence Analysis Package, version 9 (Genetics Computer Group, Madison, WI). Phylogenetic analyses were performed using the PHYLIP package, version 3.5c [Felsenstein, 1993]. Distance matrices from nucleotide sequence alignments were determined using the DNADIST program utilizing the Kimura "2-parameter" model. Phylogenetic trees were generated using FITCH. The robustness of the trees was determined by bootstrap resampling of the multiple-sequence alignments (1,000 sets) with the

TABLE I. Nucleotide and Deduced Amino Acid Identity Between Isolates of HEV Over 371 Base (123 Amino Acid) Open Reading Frame 1 Fragment

Nucleotide identity														
G1	84.4	84.1	81.9	82.5	78.4	77.9	77.6	78.2	77.9	76.6	77.6	78.2	77.4	76.6
99.2	**G2**	81.7	83.8	83.0	78.2	77.6	78.2	77.9	78.4	77.1	77.9	77.6	78.7	76.8
96.7	96.7	**It1**	84.6	86.8	77.6	77.6	77.1	77.4	77.4	76.3	77.6	77.4	77.6	78.4
98.4	97.6	96.7	**U1**	91.9	75.5	74.9	75.2	75.2	75.7	75.2	76.6	75.5	76.0	76.6
99.2	98.4	97.6	99.2	**U2**	75.2	75.4	75.2	75.4	76.0	74.9	75.7	75.7	76.3	77.6
90.2	90.2	92.7	91.9	91.1	**B1**	98.7	94.6	94.6	94.9	94.3	94.3	96.0	94.6	79.0
90.2	90.2	92.7	90.2	91.1	98.4	**B2**	93.8	93.8	94.1	93.5	93.5	95.1	93.8	78.4
88.6	88.6	91.1	90.2	89.4	98.4	96.7	**C1**	97.8	98.1	96.8	92.7	91.6	97.3	79.8
89.4	89.4	91.9	91.1	90.2	99.2	97.6	99.2	**C2**	98.7	97.6	93.8	91.6	98.4	79.5
89.4	89.4	91.9	91.1	90.2	99.2	97.6	99.2	100	**C3**	97.3	93.5	91.9	98.1	79.5
88.6	88.6	91.9	90.2	89.4	98.4	96.7	99.2	99.2	99.2	**C4**	93.0	91.9	97.0	78.7
89.4	89.4	91.9	91.1	90.2	99.2	97.6	97.6	98.4	98.4	97.6	**I1**	91.4	93.3	79.2
87.8	87.8	90.2	89.4	88.6	97.6	95.9	95.9	96.7	96.7	95.9	96.7	**I2**	91.6	78.4
89.4	89.4	91.9	91.1	90.2	99.2	97.6	99.2	100	100	99.2	98.4	96.7	**P1**	78.7
91.9	91.9	94.3	93.5	92.7	95.9	95.1	94.3	95.1	95.1	94.3	95.1	93.5	95.1	**M1**
Amino acid identity														

Isolates represented are Burmese (B1, B2), Chinese (C1, C2, C3, C4), Indian (I1, I2), Pakistan (P1), Mexican (M1), United States (U1, U2), Greek (G1, G2), Italian (It1).

programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE. Bootstrap values of higher than 70% are regarded as providing evidence for a phylogenetic grouping. The final trees were produced using RETREE (PHYLIP) with the midpoint rooting option and the graphical output was created with TREEVIEW (Page, 1996).

Abbreviations used for the various HEV isolates and the GenBank accession numbers or referenced sequences utilized in the analyses are given below. Full length HEV sequences are: Burmese isolates, B1 (M73218), B2 (D10330); Chinese isolates, C1 (D11092), C2 (L25547), C3 (M94177), C4 (D11093); Indian isolates, I1 (X98292), I2 (X99441); Pakistan isolate, P1 (M80581); Mexican isolate, M1 (M74506); United States isolates, U1 (AF060668), U2 (AF060669). Partial HEV sequences are: Swine isolate, S1 (AF011921); New Chinese isolates, Cn1 (AF082093), Cn2, Cn3 [Hsieh et al., 1998]. Abbreviations and GenBank accession numbers for the ORF1 sequences reported here are: Italian, It1 (AF110387); Greek1, G1 (AF110388); and Greek2, G2 (AF110389). The Abbreviations and GenBank accession numbers for the ORF2 sequences are: Italian, It1 (AF110390); Greek1, G1 (AF110391); and Greek2, G2 (AF110392).

RESULTS

Sequence Comparisons

Comparison of the nucleotide sequence from a 294-base pair PCR product that had been generated with primers based on the US strain of HEV had indicated that a patient from Italy was infected with an HEV strain that was divergent from other HEV isolates. This product from the 5′-end of ORF1 had a percent nucleic acid identity of 80.7%, 79.9%, and 85.8% with the prototype isolates from Burma, Mexico, and the US, respectively [Zanetti et al., 1999]. Amplification utilizing the degenerate primers from ORF1 and ORF2 gave a much more robust amplification than the US-specific primers, as indicated by a stronger ethidium bromide staining of the products (results not shown). The sequence of the product obtained with the ORF1 degenerate primer set overlapped the sequence that had been obtained with the US-specific primers and contained an additional 119 bp at the 3′-end. Sequencing of the extended ORF1 product obtained using the degenerate primers, confirmed the initial observation based on the US-specific primers. The resulting nucleotide identities compared with the three prototype isolates, were only slightly higher (1.2–3.5%) than those based on the shorter product (Table I; Zanetti et al., 1999). The divergence of the Italian isolate is also supported by the comparisons of the product from the ORF2 region of the genome that had a percent nucleic acid identity of 83.3%, 79.7%, and 87.8% with the prototype isolates from Burma, Mexico, and the US, respectively (Table II). The nucleotide identities between the prototype isolates from Burma, Mexico, and the US, range between 79.0–82.4%. Over this same region, the isolates that comprise the Burmese-like group have much higher identities of 91.2% or more.

PCR was performed on serum samples obtained from two individuals from the cohort of Greek patients with acute non-A, non-B hepatitis. Although both patients were seropositive for HEV with commercially available enzyme-linked immunosorbent assays (ELISAs), only one had been reported PCR positive, but no sequence data were reported [Psichogiou et al., 1995]. PCR products of the expected sizes were obtained with both of the degenerate primer sets upon analysis of a serum sample from the patient that was PCR positive with the Burmese-based primers. In addition, serum from the patient that had been reported previously to be PCR negative with Burmese-based primers was also found to be PCR positive with both of the degenerate primer sets. Comparisons of the ORF1- and ORF2-amplified sequences indicate that the isolates from these two patients were distinct from each other, ex-

TABLE II. Nucleotide and Deduced Amino Acid Identity Between Isolates of HEV Over 148 Base (49 Amino Acid) Open Reading Frame 2 Fragment

Nucleotide identity															
G1	87.2	87.8	84.5	85.1	85.1	84.5	82.4	82.4	83.1	83.1	82.4	83.1	82.4	83.8	81.1
100	**G2**	83.1	82.4	85.1	87.8	85.1	84.5	82.4	83.8	83.8	83.8	83.8	83.1	84.5	79.1
100	100	**It1**	87.8	85.8	85.8	83.8	83.1	82.4	83.1	83.1	82.4	83.8	81.8	82.4	79.7
98.0	98.0	98.0	**U1**	93.9	90.5	79.0	78.4	76.4	77.0	77.0	76.4	77.0	78.4	77.7	79.7
98.0	98.0	98.0	100	**U2**	91.2	82.4	80.4	79.7	80.4	80.4	79.3	80.4	81.8	81.1	81.8
98.0	98.0	98.0	100	100	**S1**	83.8	84.5	82.4	83.1	83.1	82.4	83.1	83.1	83.8	83.8
98.0	98.0	98.0	95.9	95.9	95.9	**B1**	98	94.6	95.3	95.3	94.6	96.6	97.3	93.9	82.4
95.9	95.9	95.9	93.9	93.9	93.9	98.0	**B2**	93.9	94.6	94.6	93.9	95.9	95.3	93.2	80.4
98.0	98.0	98.0	95.9	95.9	95.9	100	98.0	**C1**	98.0	98.0	96.6	96.6	91.9	96.6	81.8
98.0	98.0	98.0	95.9	95.9	95.9	100	98.0	100	**C2**	100	98.6	97.3	92.6	98.6	82.4
98.0	98.0	98.0	95.9	95.9	95.9	100	98.0	100	100	**C3**	98.6	97.3	92.6	98.6	82.4
98.0	98.0	98.0	95.9	95.9	95.9	100	98.0	100	100	100	**C4**	96.6	91.9	97.3	81.8
98.0	98.0	98.0	95.9	95.9	95.9	100	98.0	100	100	100	100	**I1**	93.9	95.9	83.8
95.9	95.9	95.9	93.9	93.9	93.9	98.0	95.9	98.0	98	98.0	98.0	98.0	**I2**	91.2	83.8
98.0	98.0	98.0	95.9	95.9	95.9	100	98.0	100	100	100	100	100	98.0	**P1**	83.1
98.0	98.0	98.0	95.9	95.9	95.9	95.9	93.9	95.9	95.9	95.9	95.9	95.9	93.9	95.9	**M1**
Amino acid identity															

Isolates represented are Burmese (B1, B2), Chinese (C1, C2, C3, C4), Indian (I1, I2), Pakistan (P1), Mexican (M1), Swine (S1), United States (U1, U2), Greek (G1, G2), Italian (It1).

hibiting 84.4% and 87.2% nucleotide sequence identity over these regions of ORF1 and ORF2, respectively. The sequences were also distinct from the Burmese-like, Mexican, US, and Italian isolates, exhibiting nucleotide identities ranging between 76.3–86.8% (Tables I and II). At the nucleotide level, the percent identities between the Greek, Italian, and US isolates ranged from 81.9% to 86.8% for the ORF1 product and from 82.4% to 87.8% for the ORF2 product. These values were lower than the percent nucleotide identities between Burmese-like isolates, which were greater than 91.2% for both ORF1 and ORF2. Many of these changes were third position substitutions that did not result in differences at the amino acid level. Thus, the amino acid differences between the isolates from Europe and the US, were therefore not significantly different than those between the Burmese-like isolates. Nonetheless, comparisons of the amino acid identities derived from the ORF1 fragment between the US, Italian, or Greek isolates and the Burmese or Mexican isolates range from 87.8% to 93.5%. These values are equal to or less than the differences between the Burmese and Mexican isolates (93.5–95.1%), indicating that the isolates from nonendemic regions are distinct from the isolates originating from endemic regions. There was less distinction between groups based on amino acid identities from the ORF2 fragment, presumably due to the short region used for comparison and the higher level of conservation observed in this region [Erker et al., 1999].

Phylogenetic Analysis With Complete Genomes

To determine more extensively the degree of relatedness between these novel isolates from Italy and Greece and other known isolates of HEV, genetic distances were determined using pairwise comparisons from multiple sequence alignments. The ORF1 and ORF2 fragments were compared with the homologous regions from 12 other human isolates of HEV from which complete genomes were available. In addition, the partial sequence from an HEV-like isolate from swine [Meng et al., 1997], was used in the ORF2 comparisons. Pairwise distances between all of these isolates for the ORF1 and ORF2 products are shown in Table III. Examination of the distance matrices demonstrates that there was considerable evolutionary distance between the three isolates of HEV from Europe and the other isolates of HEV. In contrast, the distances calculated for the Burmese-like isolates implied a close association between the isolates originating from Asia. A similar relationship was also observed between the isolates derived from the US, as indicated by the shaded portions in Table III. Within the Burmese-like group, the maximum distances calculated from the ORF1 and ORF2 products were 0.0902 and 0.0927 nucleotide substitutions per base, respectively. Within the US group, similar minimum distances of 0.0849 and 0.1001 were observed for ORF1 and ORF2, respectively. In contrast, any comparison between the US, Mexican, and Burmese groups yielded distances that were much greater than the distances between isolates within the same group. The minimum distance observed between the Mexican, US, and Burmese groups was 0.2487 for ORF1 and 0.1879 for ORF2. The minimum distance between the isolates from Europe and the isolates from the Burmese-like and Mexican isolates was greater than these values, consistent with them not being grouped with these isolates. The range of genetic distances between the European isolates and the US isolates was 0.1453–0.2077 for ORF1 and 0.1334–0.2002 for ORF2. These distances were slightly smaller than the distances between the three original prototype strains of HEV; however, the distances are still greater than the intra-group distances observed within the US or Burmese groups. The Italian isolate was most closely related to the US isolates, exhibiting

TABLE III. Genetic Distances Calculated From Pairwise Alignments of the Open Reading Frame (ORF) 1 and ORF2 Fragments

ORF1															
G1	0.1739	0.1807	0.2077	0.2016	nd	0.2644	0.2726	0.2777	0.2688	0.2726	0.2927	0.2758	0.2688	0.2803	0.2933
0.1400	**G2**	0.2120	0.1827	0.1935	nd	0.2688	0.2771	0.2706	0.2732	0.2655	0.2855	0.2726	0.2771	0.2617	0.2874
0.1353	0.1926	**It1**	0.1706	0.1453	nd	0.2688	0.2771	0.2861	0.2809	0.2809	0.2973	0.2765	0.2809	0.2771	0.2667
0.1757	0.2002	0.1334	**U1**	0.0849	nd	0.2688	0.3172	0.3147	0.3132	0.3052	0.3140	0.2927	0.3092	0.3012	0.2920
0.1665	0.1657	0.1583	0.0632	**U2**	nd	0.3125	0.3052	0.3147	0.3092	0.3012	0.3180	0.3045	0.3052	0.2973	0.2765
0.1665	0.1334	0.1568	0.1002	0.0931	**S1**	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
0.1793	0.1699	0.1879	0.2491	0.2054	0.1860	**B1**	0.0136	0.0558	0.0556	0.0528	0.0586	0.0584	0.0414	0.0556	0.2568
0.2065	0.1793	0.1976	0.2597	0.2334	0.1784	0.0206	**B2**	0.0646	0.0644	0.0615	0.0674	0.0672	0.0500	0.0644	0.2650
0.2065	0.2054	0.2065	0.2880	0.2426	0.2043	0.0559	0.0634	**C1**	0.0219	0.0191	0.0330	0.0762	0.0882	0.0274	0.2461
0.1966	0.1870	0.1966	0.2770	0.2322	0.1946	0.0485	0.0559	0.0206	**C2**	0.0136	0.0246	0.0643	0.0879	0.0163	0.2487
0.1966	0.1870	0.1966	0.2770	0.2322	0.1946	0.0485	0.0559	0.0206	0.0000	**C3**	0.0274	0.0672	0.0849	0.0191	0.2487
0.2076	0.1889	0.2076	0.2896	0.2439	0.2054	0.0562	0.0637	0.0349	0.0137	0.0137	**C4**	0.0731	0.0850	0.0302	0.2606
0.1966	0.1870	0.1879	0.2770	0.2322	0.1946	0.0344	0.0416	0.0346	0.0274	0.0274	0.0347	**I1**	0.0907	0.0701	0.2530
0.2065	0.1966	0.2154	0.2597	0.2154	0.1956	0.0275	0.0490	0.0856	0.0778	0.0778	0.0860	0.0632	**I2**	0.0879	0.2650
0.1879	0.1784	0.2054	0.2676	0.2232	0.1860	0.0629	0.0705	0.0346	0.0136	0.0136	0.0277	0.0414	0.0927	**P1**	0.2600
0.2280	0.2546	0.2466	0.2426	0.2143	0.1899	0.2043	0.2322	0.2120	0.2043	0.2043	0.2154	0.1870	0.1879	0.1956	**M1**
ORF2															

nd, not determined.
Isolates represented are Burmese (B1, B2), Chinese (C1, C2, C3, C4), Indian (I1, I2), Pakistan (P1), Mexican (M1), Swine (S1), United States (U1, U2), Greek (G1, G2), Italian (It1). Shaded cells indicate the shorter distances observed within the US or Burmese-like groups.

distances of 0.1453 for the ORF1 of HEV-US2 and 0.1583 for the ORF2 of HEV-US1. Interestingly, the two isolates from the two Greek patients were genetically distinct from each other, having distances of 0.1739 and 0.1400 for ORF1 and ORF2, respectively.

The relative evolutionary distances between the viral sequences analyzed were readily apparent upon inspection of the unrooted phylogenetic trees generated from the pairwise distances (Fig. 1), where the branch lengths were proportional to the relative genetic relationships between the isolates. The phylogenetic trees based on alignments of either ORF1 (Fig. 1A) or ORF2 (Fig. 1B) sequences were similar in overall topology. The Burmese-like isolates and the Mexican isolate represent major branches at one end of the tree. The human US isolates form a distinct group distal to the Mexican and Burmese isolates. The swine HEV-like sequence from ORF2 is closely related to the US human isolates. The three European isolates form three additional distinct branches, with the Italian isolate being most closely related to the US isolates. However, the evolutionary distance between the closest US isolate and the Italian isolate is much greater than the maximum evolutionary distance observed between the most distant Burmese-like isolates, suggesting that the Italian isolate is not a member of the US group. The lack of homology between the two Greek isolates is apparent from the distinct branch points and branch lengths associated with these two isolates. In addition, the branch length to the nearest isolate again is much greater than the maximum distance observed between the most distant Burmese-like isolates. These results are consistent with the conclusions based on differences in percent identities observed between isolates. Although alternative trees with minor differences in branching for G1 and G2 are observed, the major branch points have bootstrap values of greater than 70%, providing statistical support for the groupings.

Phylogenetic Analysis With Novel Asian Isolates

Partial sequences from several HEV isolates from Taiwan and the Liaoning Province in China have recently been described [Hseih et al., 1998; Wang et al., 1999]. Because the reported sequences of these isolates are contained within the ORF1 fragment generated with the degenerate ORF1 primers, phylogenetic analyses including these sequences were performed (Fig. 2). The fragment of the isolate from Liaoning Province is 129 base pairs shorter that the segment used for the analysis shown in Figure 1A. However, as seen in Figure 2A, a tree with the same general topology and bootstrap support is generated when using the shorter sequence. The European isolates, and the US isolates, are clearly distinct from the new Chinese isolate. These results suggest that at least seven major branches may exist among the isolates of HEV. The sequence reported for the two isolates from Taiwan were only 92 nucleotides long [Hseih et al., 1998]. A phylogenetic analysis using these additional sequences is shown in Figure 2B. Because the length of sequence used for comparison was short, the diversity in branch lengths is not as comparable as in the previous analyses (Figs. 1 and 2A), especially within the US and Burmese groups. However, the new Chinese isolates are distinct from the European isolates, as well as the US, Mexican, and Burmese isolates. In addition, the new Chinese isolates appear to form a unique group as supported by the significant bootstrap value. Sequence data from several additional HEV isolates from Taiwan has recently been described [Wu et al., 1998]. These fragments represent sequence homologous to a region of HEV in ORF1 from nucleotide positions 4,545–4,754 in the Burmese sequence and were found to have a

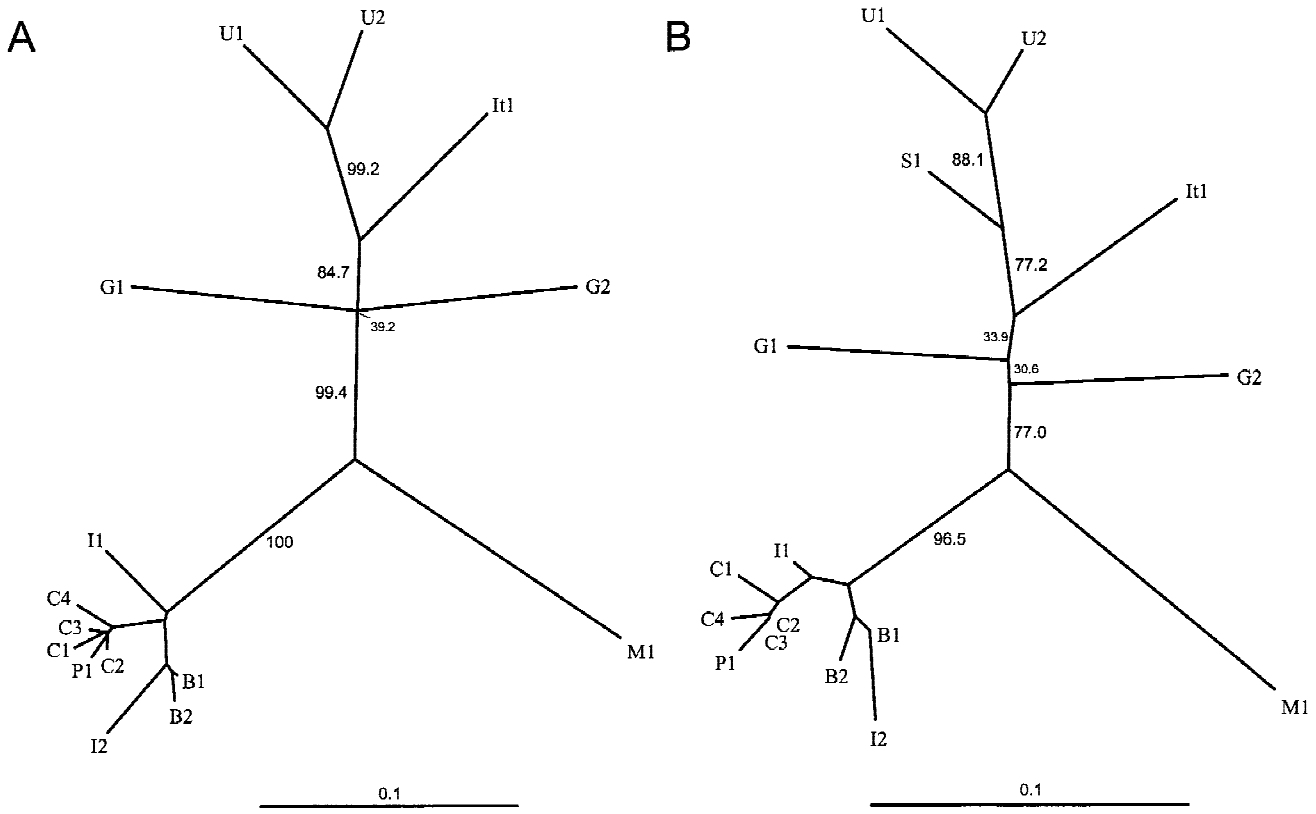

Fig. 1. Unrooted phylogenetic tree depicting the relationship of nucleotide sequences over **(A)** a 371-base fragment of open reading frame (ORF) 1 and **(B)** a 148-base fragment from ORF2. Branch lengths are proportional to the evolutionary relationship between sequences. The scale representing nucleotide substitutions per position is shown. The internal node numbers indicate the bootstrap values as a percentage of trees obtained from 1,000 replicates. Isolates represented are Burmese, B1, B2; Chinese, C1, C2, C3, C4; Pakistan, P1; Indian, I1, I2; Mexican, M1; Swine, S1; United States, U1, U2; Greek, G1, G2; Italian, It1.

close relationship with the same region isolated from two patients from the Guangzhou region in China [Huang et al., 1995]. Comparisons of these nucleotide sequences from the Guangzhou isolates had indicated that they are distinct from the US, Burmese, and Mexican isolates and could represent a distinct group of HEV [Schlauder et al., 1998]. Because there is no common region of comparison with the latter group of Asian isolates and the other isolates from Taiwan, it is unclear if these isolates are from the same group.

DISCUSSION

Three novel isolates of HEV have been identified in serum from patients with acute hepatitis infections that have not traveled to regions typically considered endemic for HEV. These new isolates are phylogenetically distinct from each other, and from other human strains isolated from or linked to travel to endemic areas. These findings expand on the recent discovery of the novel strain of HEV from an acute hepatitis infection in a patient from the US, who had no recent travel history to endemic areas [Kwo et al., 1997; Schlauder et al., 1998]. The results represent a key development in our understanding of the heterogeneity of HEV isolates and their role in the global epidemiology and potential pathogenesis of HEV infection.

The human isolate of HEV from the US is as divergent from the Burmese and Mexican strains as the Burmese and Mexican strains are from each other. Examination of the phylogenetic distances indicates that the Burmese-like strains are clustered in close proximity to the prototype Burmese strain. A similar genetic relationship is observed between the two US human isolates of HEV and the lone swine isolate from the US. The three European isolates are more closely related to the US group by percent identity and genetic distance than to the Mexican or Burmese-like isolates. However, both the percent identities and the genetic distances between the isolates from Europe and the US are greater than the distances and identities between any of the isolates within the Burmese-like group or within the US group. Therefore, based on the maximum diversity within the US and Burmese groups, the European isolates are not close enough in distance to each other or to any other isolate to be considered a member of any other genotype. These observations provide tentative support for the classification of these isolates into three distinct genotypes (Italian, Greek 1, and Greek 2), all unique from the US, Burmese, and Mexican isolates. The most genetically diverse and geographically dispersed isolates within the Burmese group exhibit much less diversity than the two Greek

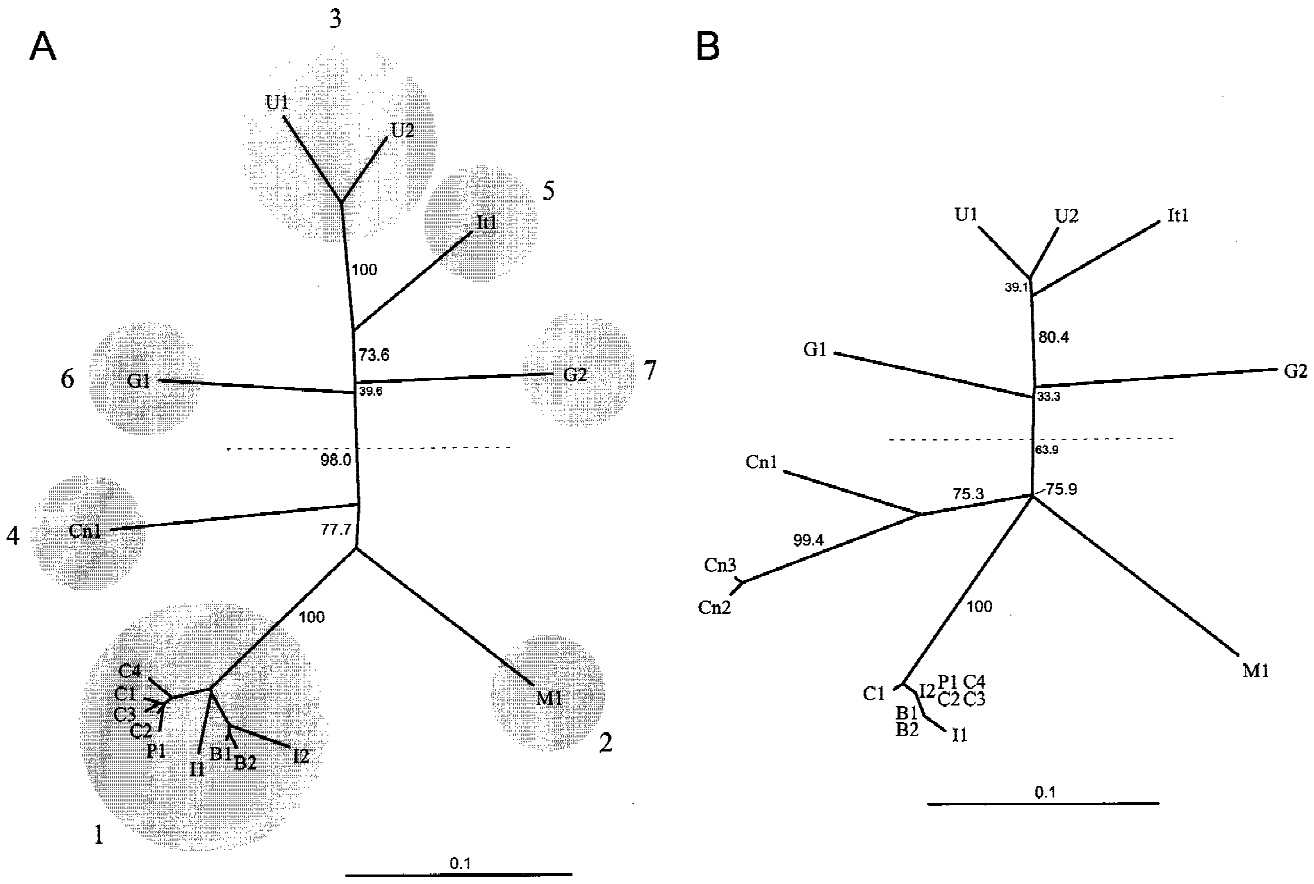

Fig. 2. Unrooted phylogenetic tree depicting the relationship of nucleotide sequences over **(A)** a 242- and **(B)** a 92-base fragment from open reading frame (ORF) 1. Branch lengths are proportional to the evolutionary relationship between sequences. The scale representing nucleotide substitutions per position is shown. The internal node numbers indicate the bootstrap values as a percentage of trees obtained from 1,000 replicates. Isolates represented are Burmese, B1, B2; Chinese, C1, C2, C3, C4; Pakistan, P1; Indian, I1, I2; Mexican, M1; Swine, S1; United States, U1, U2; Greek, G1, G2; Italian, It1; New Chinese, Cn1, Cn2, Cn3. The dashed line indicates a potential division between isolates from endemic (bottom) and nonendemic (top) regions. The number adjacent to the shaded circle indicates the genotypic designation.

isolates. Although the origin of infection of the two Greek patients cannot be determined, the extent of diversity is unexpected. It is unclear if the difference between these two isolates represents a true genetic diversity between native isolates from within one region or indicates that the source of infection originated from geographically distinct areas.

The original generalizations based on the initial discoveries of the prototype Burmese and Mexican strains of HEV and the subsequent identification of a number of closely related isolates of the Burmese strain, lead to the classification of "Old World" and "New World" forms of the virus. These definitions were useful, because the genomic analyses of the isolates represented the boundaries with respect to the genetic diversity exhibited by HEV at that time [Reyes et al., 1991]. However, these boundaries now need to be reevaluated. The identification of the US isolates has expanded on the "New World" group to include isolates in addition to the Mexican isolate. The identification of novel Asian isolates that diverge from other Burmese-like isolates and the identification of the novel isolates of HEV in Europe lead to a further expansion of the diversity within the "Old World" group, overlapping with the "New World" isolates from Mexico and the US. It is interesting to note the relative phylogenetic distribution of the new isolates of HEV from Europe and China. The variants of HEV isolated from the patients from areas traditionally considered nonendemic, group to one side of the phylogenetic tree. The isolates from endemic regions group to the other extreme of the tree. Potentially, the isolates could be classified into two major groups: the nonendemic isolates consisting of four subgroups (US, Italian, Greek 1, and Greek 2) and the endemic isolates consisting of the subgroups Mexican, Burmese-like, and new Chinese isolates. The fact that the novel HEV isolates from the US and Europe are not derived from areas considered classically endemic for HEV and that these isolates, as a group, are distinct from the isolates from endemic areas, could suggest a potential divergent evolutionary relationship between these groups. The identification of additional isolates will be needed to determine if such an association can be supported.

A numerical nomenclature for HEV genotypes has been proposed [Erker et al., 1999; Wang et al., 1999].

Based on the phylogenetic analyses with the additional European isolates, the following genotypic designations can be made: the Burmese-like group (genotype 1), the lone Mexican isolate (genotype 2), the two human isolates of from the US (genotype 3), the new Chinese isolates from Taiwan and the Liaoning Province of China (genotype 4), the Italian isolate (genotype 5), the Greek isolate 1 (genotype 6), and the Greek isolate 2 (genotype 7). The swine analogue of US HEV could be grouped with genotype 3. In addition the related Chinese isolates from Guangzhou and Taiwan could potentially represent additional members of genotype 4 or represent an eighth genotype.

The serologic implications of these findings remain to be determined. ELISAs derived from the Burmese and Mexican sequences proved inadequate in the diagnosis of acute hepatitis of one of the patients infected with the US isolate of HEV. A US strain-specific ELISA was required to determine the IgM status in the patient. In addition, preferred reactivity to US strain-specific ELISAs was reported for the other patient infected with the US strain of HEV [Schlauder et al., 1998]. Most recently, the requirement of a US-specific ELISA for the determination of antibody status in experimental infections of cynomolgus macaques with the US strain of HEV has also been reported [Erker et al., 1999]. A lack of IgG reactivity with Burmese-based ELISAs was also noted for several patients from China infected with genotype 4 [Wang et al., 1999]. The patients infected with the three European strains of HEV all showed both IgM and IgG reactivity with Burmese-based ELISAs. However, it remains to be determined if the use of strain-specific ELISAs based on these European sequences could result in an increase in sensitivity. It is therefore possible that the presence of acute HEV infection in various European populations is being underestimated due to the lack of appropriate reagents for detection of strain-specific IgM class antibodies. An ELISA capable of detecting all known strains of HEV would fill this need.

HEV is transmitted primarily by an oral-fecal route, frequently by fecal contamination of the drinking water supply. The possibility of zoonotic infections from pigs to humans was originally postulated following the experimental infection of pigs with a human strain of HEV [Balayan et al., 1990]. Studies from other domestic livestock have supported the existence of an animal reservoir for HEV [Clayson et al., 1996]. The identification of HEV-related sequence from swine herds in the US [Meng et al., 1997] and the observation that the sequence is most closely related to the human isolate of HEV from the US [Schlauder et al., 1998] suggested that zoonotic infection between pigs and humans may occur. The relatedness between a US swine isolate and the US human isolates and the identification of unique isolates from nontravelers in Europe suggest the possibility that human and animal HEV strains may also co-exist in other geographic regions.

The discovery of novel HEV variants from geographic areas not considered endemic for HEV, is important in understanding the worldwide distribution of HEV infection and underscores the need for increased awareness of HEV as a cause of acute hepatitis among nontravelers. Native strains of HEV in Europe may be responsible for some cases of acute hepatitis that are currently not linked to infection with HEV. This could be due to a lack of consideration of acute hepatitis E infection in individuals who do not travel to endemic regions and would suggest a potential need for evaluating such patients with commercially available ELISAs for possible hepatitis E infection. In addition, if serologic analysis is performed, the possibility of decreased or lack of antibody reactivity due to the requirement of strain specific reagents could account for misdiagnosis. The existence of native European strains may also help explain the increased seroprevalence of HEV despite the rarity of HEV-associated hepatitis cases. Additional studies on the prevalence of HEV-related sequences or HEV-reactive antibodies in swine, as well as other animal species, will be helpful in discerning the potential impact they may have on endemic and sporadic HEV.

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